The *Phytolacca americana*-derived naturally occurring ribosome inhibitory protein pokeweed antiviral protein (PAP) is an N-glycosidase that catalytically removes a specific adenine residue from the stem loop of ribosomal RNA. We have employed molecular modeling studies using a novel model of PAP-RNA complexes and site-directed mutagenesis combined with bioassays to evaluate the importance of the residues at the catalytic site and a putative RNA binding active center cleft between the catalytic site and C-terminal domain for the enzymatic deadenylation of ribosomal RNA by PAP. As anticipated, alanine substitutions by site-directed mutagenesis of the PAP active site residues Tyr72, Tyr123, Glu176, and Arg179 that directly participate in the catalytic deadenylation of RNA resulted in greater than 3 logs of loss in depurinating and ribosome inhibitory activity. Similarly, alanine substitution of the conserved active site residue Trp206, which results in the loss of stabilizing hydrophobic interactions with the ribose as well as a hydrogen bond to the phosphate backbone of the RNA substrate, caused greater than 3 logs of loss in enzymatic activity. By comparison, alanine substitutions of residues 28KD28, 80FE81, 113SR112, 166Fl167 that are distant from the active site did not significantly reduce the enzymatic activity of PAP. Our modeling studies predicted that the residues of the active center cleft could via electrostatic interactions contribute to both the correct orientation and stable binding of the substrate RNA molecule in the active site pocket. Notably, alanine substitutions of the highly conserved, charged, and polar residues of the active site cleft including 48KY49, 67RR68, 86NN70, and 90FND92 substantially reduced the depurinating and ribosome inhibitory activity of PAP. These results provide unprecedented evidence that besides the active site residues of PAP, the conserved, charged, and polar side chains located at its active center cleft also play a critical role in the PAP-mediated depurination of ribosomal RNA.

Pokeweed antiviral protein (PAP)\(^1\) is a 29-kDa naturally occurring protein isolated from the leaves of the pokeweed plant, *Phytolacca americana* (1–3). PAP belongs to a family of plant ribosome-inactivating proteins (RIPs) that catalytically depurinate ribosomal RNA (3, 4). The enzymatic activity of PAP has been shown to be the specific cleavage of the glycosidic bond of a single adenine residue (\(A_{4324}\) of the tetraloop sequence GAGA) that is located in the highly conserved sarcin/ricin stem loop (4–7) and is found in both eukaryotic rRNA (28 S) and in prokaryotic rRNA (23 S). The ribosomes depurinated by PAP in this manner are unable to interact with elongation factors 1 and 2 (8, 9), and thus the protein synthesis is irreversibly inhibited at the translocation step (7, 8).

The x-ray structure of PAP has been determined previously and is composed of eight \(\alpha\) helices and a \(\beta\) sheet consisting of six strands (15–17). The refined crystal structure of PAP suggests that the protein can be divided into three domains: the N-terminal domain (residues 1–69, PAP numbering), the central domain (residues 70–179), and the C-terminal domain (residues 180–262). All of the highly conserved catalytic site residues (Tyr72, Tyr123, Glu176, and Arg179) are located in the central domain. A deep cleft (“active center cleft”) at the interface between the central and C-terminal domains forms the putative substrate-binding site (16).

Structural studies involving complexes of PAP with various ligands (adenine, formycin, and pteoric acid) have helped clarify the nature of the substrate binding site of PAP (15, 17) and provided valuable information concerning its substrate specificity. However, these studies have largely been limited to single nucleotides or nucleotide analogs in the active site, and details of how PAP binds a larger RNA fragment of the ribosome remain unknown. Recent modeling studies of another member of the RIP family (ricin) have also been useful in suggesting binding modes of RNA fragments in the active site (18). Based on structural, mutagenesis, and biochemical studies of several RIPs (ricin-A chain, trichosanthin, and momorcharin), it has been proposed that the amino acids Tyr72 and Tyr221 (PAP numbering) have the role of sandwiching the susceptible adenine ring of ribosomal RNA into the energetically favorable stacking conformation (19, 20). Subsequently, the side chain of Arg179 can protonate the N-3 atom of the adenine base, whereas Glu176 stabilizes a positive oxocarbonium transition state (15, 20). Huang et al. (21) have recently proposed that the N-7 atom of the adenine base can also be protonated by an acidic residue such as Asp92 (PAP numbering) in trichosanthin and momorcharin.

We have recently reported the expression of biologically active recombinant PAP in *Escherichia coli* (22). The biological activity of recombinant PAP was virtually identical to that of native PAP purified from the pokeweed plant (22). The availability of recombinant PAP provides a unique opportunity for structural-activity relationship (SAR) analyses. Furthermore, we have refined the x-ray structure of PAP (17) and established a novel model of the PAP-RNA complex using the coordinates of PAP (Protein Data Bank access code 1qcg), PAP complexed...
with formycin 5'-monophosphate (Protein Data Bank access code 1pag), as well as the coordinates of the ribosomal RNA stem loop from the crystal structure of the 29-nucleotide RNA fragment (Protein Data Bank access code 430d) of rat 28 S ribosomal RNA, which contains the sarcin/ricin loop.

In the present SAR study, we employed molecular modeling studies using our model of PAP-RNA complexes and site-directed mutagenesis with bioassays to evaluate the importance of the residues at the catalytic site and a putative RNA binding active center cleft between the catalytic site and C-terminal domain for the enzymatic deadenylation of ribosomal RNA by PAP. As anticipated, alanine substitutions by site-directed mutagenesis of the PAP active site residues Tyr72, Tyr123, Glu176, and Arg179 that directly participate in the catalytic deadenylation of ribosomal RNA resulted in greater than 3 logs of loss in degrading and ribosome inhibitory activity. Similarly, alanine substitution of the conserved active site residue Trp108 which results in the loss of stabilizing hydrophobic interactions with the ribose as well as a hydrogen bond to the phosphate backbone of the RNA substrate, caused greater than 3 logs of loss in enzymatic activity. By comparison, alanine substitutions of residues 28KD29, 80FE81, 111SR112, and 166PL167 that are distant from the active site did not significantly reduce the enzymatic activity of PAP. Our modeling studies predicted that the residues of the active center cleft could via electrostatic interactions contribute to both the correct orientation and stable binding of the substrate RNA molecule in the active site pocket. Notably, alanine substitutions of the highly conserved, charged, and polar residues of the active site cleft including 49KY49, 67RR68, 69NN70, and 90FND92 substantially reduced the degrading and ribosome inhibitory activity of PAP. Our findings presented herein provide unprecedented experimental evidence that besides the catalytic site residues, the conserved charged and polar side chains located at the active site cleft of PAP also play a critical role in the catalytic removal of the adenine base from target ribosomal RNA substrates.

**MATERIALS AND METHODS**

**Molecular Modeling**—We first modeled the interaction of PAP with the single-stranded RNA heptamer GAGAGGA, which contains the target sequence for PAP. The initial position of GAGAGGA single-stranded RNA was built manually using RNA coordinates generated by InsightII (33). The position of the adenine base in PAP active site pocket (Protein Data Bank access code 1QCI) (17) was used as a guide to properly position the second adenine in the modeled RNA heptamer. Major steric collisions with PAP were removed by manually adjusting the torsion angles of the phosphate backbone. The RNA heptamer was roughly positioned within the long concave region on the surface of PAP. This general position of the RNA heptamer was used as a starting point for subsequent docking trials. We created a definitive binding set of PAP residues in the active site pocket to move as a 3.5 Å shell around the manually docked RNA substrate during energy minimization. The number of final docking positions was set to 10, although finally only 2–4 promising positions were identified. The calculations used a consistent van der Waals force field in the Discovery program and a Monte Carlo strategy in the Affinity program. Each energy-minimized final docking position of the ligand was evaluated using the interactive score function in the Ludi module. During RNA docking four torsion angles in phosphate backbone near the bound alanine were freed to increase the volume of conformational search. The final binding position of the RNA heptamer was determined based on the evaluation of favorable binding interactions using the Ludi score function. Ludi score included the contribution of the loss of translational and rotational entropy of the RNA fragment, number, and quality of hydrogen bonds and contributions from minor and major groove interactions.

We next modeled the interaction of PAP with a 29-nucleotide RNA fragment of rat 28 S RNA. The initial position of the ribosomal RNA stem loop in the PAP active site was built manually. This was done using the coordinates of PAP (Protein Data Bank access code 1pag) (17), PAP complexed with FMP (an adenosine analog, Protein Data Bank access code 1pag) (15) and the coordinates of the ribosomal RNA stem loop, which contains the sarcin/ricin loop, from the crystal structure of the 29-nucleotide RNA fragment (Protein Data Bank access code 430d) (23). To place the RNA fragment in the PAP active site, the nucleotide A15 (analogous to A4324 in eukaryotic rRNA) was first removed from the coordinates of the RNA fragment. Because FMP binds to the PAP in a conformation to be similar to that of the targeted adenine of the rRNA fragment, this position was used as a guide to place the resulting 28-nucleotide RNA fragment (residues 1–14, 16–29). The possible positions of this RNA fragment in the PAP active site were manually explored by allowing only rigid body movements under the following constraints: the 5'-phosphate group of G15 should be within a bonding distance from the 3'-OH of FMP, and the 3'-OH of G14 should be within a bonding distance from the 5'-phosphate group of FMP. Under such constraints, there was only one general position of the RNA structure that avoided major steric interference with PAP. Once the RNA fragment was positioned, the coordinates of the A15 nucleotide were reinserted so that it matched the position of FMP. This general position of RNA in the active site of PAP was then used as a starting point for further modeling studies.

To refine this initial position and to explore other possible positions, the initial model was used to perform fixed docking using the Docking module in InsightII employing the CVFF forcefield (33). The parameters used in this docking included searching for five unique structures: 1000 minimization steps for each structure, energy range of 10.0 kcal/mol, maximum translation of the ligand of 10 Å, and an energy tolerance of 1500 kcal/mol. During the minimization steps of the docking procedure, only the stem loop residues 13–18 were allowed to be flexible, whereas the residues 1–12 and 19–29 were held fixed. In addition, several distance and torsion restraints were applied to the 13–18 GC base pair to maintain this interaction.

**Bacterial Strains and Construction of Mutants**—Recombinant wild-type PAP (phosphate-buffered saline-PAP) was obtained by subcloning the PAP-I gene (amino acids 22–313) into the pBluescript SK expression vector (22). Uracil-containing template of PAP was obtained by transforming E. coli CJ236 with the recombinant plasmid phosphate-buffered saline-PAP. The oligonucleotides used for site-directed mutagenesis were synthesized by BioSynthesis Inc. (Lewisville, TX). Site-directed mutagenesis procedure was as described in the manufacturer’s manual (Mutagen M13 in vitro mutagenesis kit; Bio-Rad). DNA sequencing was carried out by the method of Sanger et al. (24) following the manufacturer’s instructions (U. S. Biochemical Corp.). Fine chemicals and restriction enzymes were purchased from Roche Molecular Biochemicals.

**Expression and Purification of Mutants**—Wild-type and mutant recombinant(r) PAP proteins were expressed in E. coli: MV1190 as inclusion bodies, isolated, solubilized, and refolded as described previously (22). The refolded proteins were analyzed by SDS-12% polyacrylamide gel electrophoresis (PAGE). Protein concentrations were determined from the gel using bovine serum albumin as a standard.

**Immunoblot Analysis of rPAP Mutants**—The protein samples were resolved by electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad) using the Bio-Rad trans-blot apparatus, as described previously (22). The membrane was immunoblotted using rabbit anti-PAP serum (1:2000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the primary and secondary antibodies, respectively. The blot was developed using 3,3-diaminobenzidine chromogenic indicator for peroxidase activity.

**Aniline Cleavage Assays of Ribosomal RNA Depurination**—5 μg of E. coli 16 S and 23 S tRNA (Roche Molecular Biochemicals) or 30 μg of total ribosome prepared from the rabbit reticulocyte-rich whole blood (25) were incubated with either 5 or 25 μg of wild-type or mutant rPAP proteins in 50 μl (final volume) of binding buffer (25 mM Tris-HCl, pH 7.8, 10 mM KC1, 5 mM MgCl2, 2% glycerol) at 37 °C for 1 h. The RNA was extracted with phenol:chloroform (24:24), precipitated with ethanol, and treated with 20 μl of 1 M aniline acetate (pH 4.5) for 30 min on ice. The RNA was precipitated with ethanol, electrophoresed in a 6% urea/polyacrylamide gel, and stained with ethidium bromide as described previously (22).

**Adenine Release Assays**—The release of adenine from ribosome was measured using the Adenine Release Assay kit from New England BioLabs (Beverly, MA). The reaction contained 10 mM Tris-base, 150 mM KCl, pH 7.8, 10 mM MgCl2, and 50 μl of the sample was added. The reaction was started by adding 100 μl of HPLC running buffer (50 mM NH4HCO3, 5% methanol, pH 5.0), and 100 μl of the sample was
injected automatically into a reverse-phase Lichrospher 100RP-18E analytical column (Hawlett-Pakard, 5-mm particle size, 250 \times 4 \text{ mm}) that was equilibrated with HPLC running buffer. The detector wavelength was set at 280 nm, and the sample was eluted under isocratic conditions at a flow rate of 1.0 ml/min as described before (14). Controls included (a) samples containing untreated ribosomal RNA and (b) samples without ribosomal RNA. The adenine standard was purchased from Sigma. A calibration curve was generated to establish the linear relationship between the absolute peak area and the concentrations of adenine in the tested samples. Adenine at final concentrations of 0.1, 0.5, 1.0, 2.5, and 5.0 \mu M (5, 25, 50, 125, and 250 pmol/50 ml, respectively) was injected into the HPLC system for analysis, and the calibration curve was generated by plotting the absolute peak area against the concentrations of adenine as described previously (14). Unweighted linear regression analysis of the calibration curve was performed by using the CA-Cricket graph III computer program (Computer Association, Inc., Islandia, NY). Intra-assay and inter-assay accuracy and precisions were evaluated as described previously (14). Under the described chromatographic conditions, the retention time for adenine was 9.7 min, and adenine was eluted without an interference peak from the blank controls (data not shown). The lowest limit of detection for adenine was 2.5 pmol at a signal to noise ratio of \( \approx 3 \). The intra- and inter-assay coefficients of variation were less than 4%. The overall intra- and inter-assay accuracies of this method were 98.7 \pm 1.7\% (\( n = 6 \)) and 95.7 \pm 3.0\% (\( n = 6 \)), respectively.

**Cell-free Translation Assays**—Protein synthesis was assayed in a cell-free system using nuclease-treated rabbit reticulocyte lysates (Promega, Madison, WI) and luciferase mRNA, as described previously (22). In this assay, luciferase mRNA was translated in the presence or absence of increasing concentrations of wild-type or mutant rPAP proteins. Luciferase mRNA encodes for a monomeric protein of 61 kDa on SDS-PAGE. In brief, varying amounts (0.01–12,000 ng/ml) of wild-type or mutant rPAP proteins were added to the translation mixture (10 \mu l rabbit reticulocyte lysate; 0.5 \mu l of 1.0 mM methionine-free amino acids mixture; and 1.0 \mu l of [\textsuperscript{35}S]methionine, 10 \text{ Ci}/\text{ml}) to a final volume of 19 \mu l and incubated for 15 min at room temperature. Protein synthesis was initiated by adding 0.12 \mu g of luciferase mRNA in a 1.0-\mu l volume, and the incubation was continued for 2 h at 30 °C. Translation was stopped by the addition of 5% trichloroacetic acid, and the precipitated polypeptides were collected on Whatman GF/C glass microfilter filters. The incorporation of [\textsuperscript{35}S] was determined by counting the radioactivity on the filters using a liquid scintillation counter (Beckman LS 6000SC) as described previously (22). The IC\textsubscript{50} (50\% inhibitory concentration) values were calculated by nonlinear regression analysis (Prism-2 Graph Pad software, San Diego, CA) using the average values of three independent experiments. The cpm values in control sample with all the reagents added except the test sample ranged from 3 to 4 \times 10^7 \text{ cpm/ml} and were considered as 100\% incorporation when determining the percentage of control protein synthesis values for samples treated with test materials.

**RESULTS AND DISCUSSION**

**Modeling Studies of the Catalytic Active Site and RNA-binding Residues of PAP**—Our first model of PAP-RNA heptamer complex (i.e. PAP-GAGAGGA complex) indicated that the target RNA heptamer can bind very strongly to PAP via multiple interactions along the concave cleft region (Fig. 1). The central adenine base is sandwiched between Tyr\textsuperscript{72} and Tyr\textsuperscript{123} as observed previously (15, 17) and forms four hydrogen bonds with active site residues (Val\textsuperscript{73}, Ser\textsuperscript{121}, and Arg\textsuperscript{179}). There are additional stabilizing electrostatic interactions between neighboring negatively charged phosphate groups and two clusters of positively charged interactions on the PAP surface formed by Arg\textsuperscript{179} and Lys\textsuperscript{210} from one side and by Arg\textsuperscript{123} and Arg\textsuperscript{139} from the other side of the active site. The two adjacent guanines (G\textsuperscript{3} and G\textsuperscript{5}) of the bound adenine (A\textsuperscript{4}) do not have any specific interactions with PAP, whereas the other ribonucleotides interact with the active site cleft residues. These interactions help to properly position the adenine base in the PAP active site so that it can be cleaved with high efficiency.

Our second more advanced model of PAP-RNA complex involved an RNA stem loop fragment (29-nucleotide, 5'-GGUGUCUCAGUCAAGGAACCGACACCC-3') docked into the active site of PAP as shown in Fig. 2. This orientation allows the purine ring of adenine, at the position that corresponds to A\textsuperscript{15}, of the stem loop to be sandwiched between the side chains of Tyr\textsuperscript{122} and Tyr\textsuperscript{123}, as observed crystallographically (15) for the adenine analog, FMP (formycin monophosphate). The crystallographic position of FMP shows that the N\textsuperscript{6} of the adenine analog ring donating a hydrogen bond to the carbonyl oxygen of Val\textsuperscript{73} whereas N\textsuperscript{3} is accepting a hydrogen bond from the amide backbone of Val\textsuperscript{73}. Our model, however, positions A\textsuperscript{15} in a slightly different position and now has N\textsuperscript{6} donating a hydrogen bond to the carbonyl oxygen of Ser\textsuperscript{121} and is slightly more distanced from the carbonyl oxygen of Val\textsuperscript{73}. In addition, the distance between N\textsuperscript{1} and the amide...
backbone of Val73 is also slightly out of range for a hydrogen bond to occur. The active site residue Glu176 is in a position similar to what has been observed experimentally, where the negatively charged chain is under the glycosidic bond of A15 and is proposed to stabilize the positive oxocarbonium ion that develops on the ribose group in the transition state (15, 26). Another active site residue Arg179 also shows a similar conformation where it is in a position to protonate N3 of the adenine ring. Thus, the overall conformation of the PAP active site residues of our model is similar to those observed previously (18), as shown in Fig. 2.

The base stacking pattern of the model is also interesting to
As mentioned previously, A15 is sandwiched between two tyrosine residues, and G16 is stacked on top of Tyr72. The modeled positions of A15 between the tyrosine residues (Tyr72 and Tyr123) of PAP should allow A15 to be inserted into the active site of PAP and still maintain the favorable base stacking interactions that help stabilize the RNA structure. Several contacts are formed with the stem loop that appear to provide specificity for A15 and G16 of the stem loop. The majority of these contacts are formed with A15 and include hydrogen bonds with Ser121 and Arg179. The Asp92 forms a hydrogen bond with the amine and N1 of the G16 base. There are several additional binding contacts are formed between the phosphate backbone and the positively charged residues Arg67, Arg122, Arg179, and Lys210, as well as several other polar residues such as Asn70 of the active site cleft. All of the contacts that are formed between PAP and the RNA fragment are shown schematically in Fig. 3.

Both models support the notion that the active site residues Tyr72, Tyr123, Glu176, and Arg179 are directly responsible for the catalytic function of PAP. In addition, modeling studies with the RNA fragments uniquely indicate that several residues (Lys48, Arg67, Asn69, Asn70, Asp92, Arg122, and Lys210) that are not directly involved in the catalytic depurination at the active site are forming specific interactions with the RNA substrate. The model of PAP complexed with the 29-nucleotide RNA stem loop fragment indicates that the positive charge of Arg67 should enable this residue to favorably interact with the negatively charged phosphate backbone of G18 in RNA, whereas Asn69 and Asn70 interact with the phosphate backbone of G18 and ribose of G16, respectively (Fig. 3). According to our model, Asp92 interacts with the base of G16 and may therefore contribute to the binding of PAP to the tetraloop structure of RNA (Fig. 3). Our model also suggests that the side chain of Trp208 can engage in a hydrophobic interaction with the ribose of G16 and the amide backbone of Trp208 can form hydrogen bonds with the phosphate backbone of G16. Therefore, mutations involving Trp208 could result in local conformational change at the catalytic site of the PAP and disrupt these stabilizing interactions with G16.

Our models indicate that the above interactions are important for binding, orientation, and stabilization of the RNA substrate, and their mutation could therefore lead to significant loss of catalytic activity. Obviously the removal of positively charged residues will diminish the strength of binding to the negatively charged phosphate backbone of RNA and subsequently reduce the activity PAP. Besides, Asn70 forms a hydrogen bond with the catalytic site residue Arg179, and ala-
nine mutation of this residue would therefore likely affect the enzymatic activity of PAP.

Construction and Expression of PAP Mutants—Recombinant PAP mutants with alanine substitutions of the conserved catalytic site and active center cleft residues were constructed using site-directed mutagenesis techniques. Alanine substitutions were considered because the replacement of side chains with alanine would be least disruptive to the overall structure (27). Also, alanine does not impose new hydrogen bonding, sterically bulky, or unusually hydrophobic side chains (27). An amino acid alignment of PAP residues, selected for mutagenesis, with those of PAP-II, PAP-S, and ricin-A chain is shown in Fig. 4. The active site residues selected for mutagenesis are highly conserved among the various RIPs identified to date (Fig. 4A). The residues outside the active site chosen for mutagenesis are highly conserved among the PAP isoforms, but they are different from those in ricin-A chain (Fig. 4C). The positions, chemical nature, and secondary structural elements of the amino acids substituted with alanine are indicated in Table I.

Thirteen point mutants of recombinant PAP (Table I), containing single, double, or triple alanine substitutions, were constructed and expressed in the E. coli strain, MV1190, as inclusion bodies. The inclusion bodies were purified, solubilized, refolded, and analyzed by SDS-PAGE (Fig. 5A). All of the mutant proteins were expressed in yields comparable to that of the wild-type PAP (Fig. 5A). The solubilized and refolded mutants displayed a 33-kDa major protein on SDS-PAGE, were highly reactive to the anti-PAP serum and were stable under our solubilization and refolding conditions (Fig. 5B).

Inhibition of Translation in the Rabbit Reticulocyte Lysate Assays—The mutants could be roughly categorized into three major groups. The first group includes PAP mutants with alanine substitutions distant from the catalytic active site (FLP-1/28AA29, FLP-6/80AA81, FLP-8/111AA112, and FLP-10/166AA167; colored blue in Fig. 1). Based on our modeling studies we did not expect these mutants to have substantially reduced activity. The experimentally determined IC₅₀ values from cell-free translation assays confirmed that the ribosome inhibitory activity of FLP-1, FLP-6, FLP-8, and FLP-10 proteins (IC₅₀ =
were comparable with that of the wild type (IC₅₀ 5.7 ± 1.0 ng/ml; Fig. 6 A and Table I). The data from adenine release assays and aniline cleavage assays were in accordance with the translation inhibition data.

The second group of PAP mutants include those with mutations at the catalytic active site (FLP-5/71AA₇₂, FLP-9/122AA₁₂₃, FLP-11/Ala₁₇₆, FLP-12/Ala₁₇₉, and FLP-13/Ala₂₀₈; colored red in Fig. 1). Alanine substitutions at these locations were expected to affect the orientation of the substrate adenine in the active site and subsequent enzymatic cleavage of the glycosidic bond. As predicted from our modeling studies, these mutants were enzymatically inactive (Fig. 6 B and Table I). At a 5 μM concentration, wild-type PAP released 396 ± 15 pmol of adenine/μg of ribosomal RNA (Table I). By comparison, the catalytic site mutants, FLP-5, FLP-9, FLP-11, FLP-12, and FLP-13 released 9 ± 2, 12 ± 3, 7 ± 2, 14 ± 7, and 4 ± 2 pmol of adenine/μg of rRNA, respectively. Substitution of the catalytic active site residues 71LY₇₂ (FLP-5) and 122RY₁₂₃ (FLP-9), Glu₁₇₆ (FLP-11) and Trp₂₀₈ (FLP-13) have resulted in nearly complete loss (>1700-fold less active) of ribosomal depurination activity, as we expected. Arg₁₇₉ (active site residue), however, apparently plays a less important role in catalytic deenylation than the other active site residues (only 385-fold less active than the wild type).
The third group of PAP mutants included those with mutations in the active center cleft between the central and C-terminal domains, dominated by charged (Lys⁴⁸, Arg⁶⁷, Arg⁶⁸, and Asp⁹²) and polar (Asn⁶⁹ and Asn⁷⁰) side chains forming contacts with the modeled RNA substrates, (FLP-2⁴⁸AA⁴⁹, FLP-3⁶⁷AA⁶⁸, FLP-4⁶⁹AA⁷⁰, and FLP-7⁹²AA⁹²; colored green in Fig. 1). All of these mutated residues are involved in a complex network of interactions pivotal for the proper orientation of the substrate RNA. In addition, Asn⁷⁰ (FLP-4) forms a hydrogen bond with the catalytic residue Arg¹⁷⁹, and alanine substitution might lead to a functionally unfavorable conformation of the Arg¹⁷⁹ side chain (Fig. 7). Notably, mutations at the active center cleft have markedly diminished (23-, 33- 191- and 352-fold less active, respectively) the enzymatic activity of PAP (Fig. 6C and Table I). As shown in Fig. 6, the inhibition curves shifted to the right, consistent with a significant decrease in enzymatic activity. The bioassays were carried out using at least four different preparations of wild-type and mutant rPAP and yielded comparable results. Substitution of ⁴⁸KY⁴⁹ (FLP-2) at the far end of the cleft had a less pronounced effect on the catalytic activity (23-fold less active) than the substitution of the residues ⁶⁷RR⁶⁸, ⁶⁹NN⁷⁰, and ⁹⁰FND⁹² that are located closer to the catalytic site (Table I). The adenine release assays were also consistent with the protein synthesis inhibition assay results (Table I).

We next examined whether there was deadenylation of ribosomal RNA (rabbit) by the catalytic site mutants or active center cleft mutants. The rabbit rRNA depurination was determined by treating the ribosomes with wild-type or mutant proteins and subsequent purification of the rRNA and cleavage with aniline. Because aniline cleaves the sugar-phosphate backbone of RNA at depurination sites, the release of fragments from aniline-treated RNA is indicative of depurination. Our results indicated that neither the catalytic site mutants nor the active site cleft mutants have deadenylated the rRNA.
The ribosomal RNA depurination activity was also determined by treatment of *E. coli* 16 S and 23 S ribosomal RNA with wild-type and mutant proteins and subsequent cleavage of the treated RNA with aniline. Treatment of the naked rRNA (2 μg) with 2.5 μg of either the wild-type or the enzymatically active mutant proteins (FLP-1, FPL-8, and FLP-10) has released an RNA fragment of ~240 nucleotides (Fig. 8B), whereas the less active mutants, FLP-2 and FLP-3, required a 10-fold higher amount of protein (25 μg) to release the 240-nucleotide RNA fragment (Fig. 8C). On the other hand, the mutants that substantially reduced the deadenylation activity (FLP-5, FLP-7, FLP-9, FLP-11, and FLP-13) did not release the fragment even at 25 μg/ml concentration of the mutant proteins (Fig. 8D). These results are in agreement with the data obtained from adenine release assays and protein synthesis inhibition assays.

In summary, we employed molecular modeling studies using our model of PAP-RNA complexes and site-directed mutagenesis combined with bioassays to evaluate the importance of the residues at the catalytic site and a putative RNA binding active center cleft between the catalytic site and C-terminal domain for the enzymatic deadenylation of ribosomal RNA by PAP. Our findings presented herein provide unprecedented experimental evidence that in addition to the catalytic site residues, the conserved charged and polar side chains located at the active site cleft of PAP also play a critical role in the catalytic removal of the adenine base from target ribosomal RNA substrates.

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